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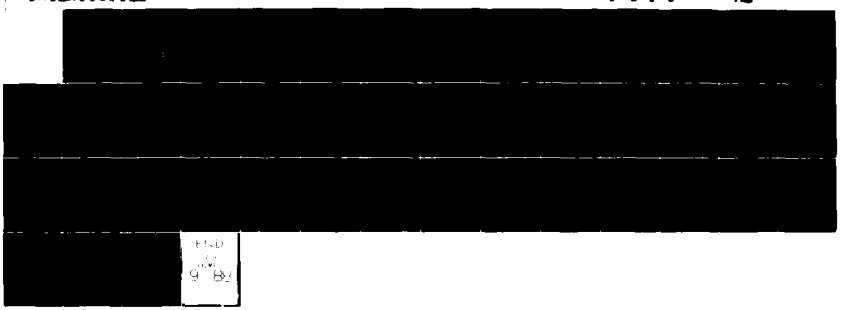

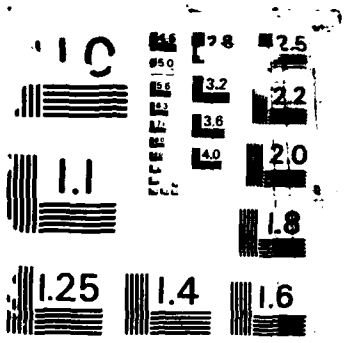

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Human Hybridomas for Exotic Antigens

Annual Report

Dr. Melvin Cohn, Ph.D.

October 1, 1987

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The broad aim of this work is to develop methods that will allow human lymphocytes to be immunized in vitro with exotic (including toxic) antigens to generate specific antibody-producing cells that can be captured as hybridomas and thereby serve as a source of specific human antibodies directed against exotic antigens. Our approach has been guided by the general finding that in vitro immunization of human peripheral blood lymphocytes (PBL) with exotic antigens is, at best, difficult. Rather than push the in vitro immunization techniques to the point where antibody is secreted in vitro (the usual assay procedure) we opted to use the hybridoma methodology to capture B-cells at an earlier stage of differentiation, before full scale antibody secretion. Thus, the focus of our efforts over the past 2 years has been to activate PBL and generate B-cells that can be captured as hybridomas.

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SUMMARY

The broad aim of this work is to develop methods that will allow human lymphocytes to be immunized *in vitro* with exotic (including toxic) antigens to generate specific antibody–producing cells that can be captured as hybridomas and thereby serve as a source of specific human antibodies directed against exotic antigens. Our approach has been guided by the general finding that *in vitro* immunization of human peripheral blood lymphocytes (PBL) with exotic antigens is, at best, difficult. Rather than push the *in vitro* immunization techniques to the point where antibody is secreted *in vitro* (the usual assay procedure) we opted to use the hybridoma methodology to capture B–cells at an earlier stage of differentiation, before full scale antibody secretion. Thus, the focus of our efforts over the past 2 years has been to activate PBL and generate B–cells that can be captured as hybridomas.

At the outset we had in hand a large body of data to show that the WI–L2–729–HF_2 tumor parent was able to generate hybridomas that secrete new (i.e., PBL–derived) immunoglobulin. Experience with mouse hybridoma systems led us to expect that new immunoglobulin and specific antibodies were synonymous. From the data we have accumulated over the past 2 years it is now reasonably clear that the human hybridoma system is very different from the mouse system. Two points stand out. First, as a general observation, there are few human B–cell tumor lines that are suitable for generating stable hybridomas, and the WI–L2–729–HF_2 still seems to be the best. Second, and more important, neither we nor others who have been working intensively in this field over the past 5 years have produced more than occasional antigen–specific hybridomas, and even fewer of these have remained stable – very few can be obtained "off–the–shelf" as is typical of published reports of mouse hybridomas. Thus, it would apperar that at present the use of hybridoma technology to study *in vitro* immunization procedures is impractical.

At this stage we face a major question as to whether continued efforts should be made with hybridoma systems, or should our efforts should be shifted to study *in vitro* immunization. The issue is complicated further by the fact that even if we did achieve our goal of efficient *in vitro* immunization, would this be of use if we could not capture these B–cells as hybridomas? Since the two issues are so closely linked, we plan to emphasize the *in vitro* immunization studies and allow work on the hybridoma system to continue with a lower priority.

The bulk of the results presented in this report represent examples taken from a large number of experiments which collectively have driven us to the conclusion that human hybridoma systems are, in their present form, inadequate to serve as a reliable means of producing monoclonal human antibodies.

FOREWORD

Citations of commercial organizations or trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Research Council (DHEW Publication No. (NIH)78-23, Revised 1987).

For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45FR46.

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1. OBJECTIVES.

The overall goal of the work being carried out under this contract is the development of methods for generating human hybridomas specific for highly toxic exotic antigens. Of primary interest is the development of methods that will enable human B-lymphocytes to be immunized *in vitro* in a way suitable for fusion with the B-cell tumor WI-L2-729-HF$_2$, thereafter enabling the capture of stable antibody-producing hybridomas. Included in the broad aims of this contract is the production of human hybridomas from immune donors, particularly in the case of volunteers immunized with attenuated vaccines and known to be producing protective antibodies against viruses of major military significance.

2. BACKGROUND.

There is no doubt that primary *in vitro* immunization of human B–cells and their capture as hybridomas represents a major technical advance and has considerable military significance. With this technology in hand it would become possible to generate antibodies against highly toxic pathogens that would otherwise be lethal *in vivo*; an exception would be that rare class of agents which lethally infect B–lymphocytes.

The human hybridoma technology has not matured with the same speed and consistency as the murine systems for reasons which remain frustratingly obscure. In a recent comprehensive review of the field James and Bell (1) have catalogued all of the published examples of human hybidomas; a the remarkable feature of this compendium is how few repeat successes come from any given laboratory. Our experience reflects that of others, namely that it is difficult to generate human hybridomas which secrete a defined specificity with a high degree of reproducibility. However, we emphasize that the technique is sufficiently important to warrant a continued vigorous effort and there is no theoretical reason to suggest that the human hybridoma system cannot be made to work.

3. EXPERIMENTAL METHODS.

3. A. Fusion method.

3. A. i. Preparation of PEG.

Weigh 1 g of PEG (MW 4000 from EM Science) into a glass tube and autoclave to sterilize. Cooled PEG is melted immediately prior to use by heating in a boiling water bath, then transfer to 56° water bath. Prepare fresh a 25% (v/v) solution of DMSO in RPMI 1640 at room temperature. Add 1 ml of 25% DMSO to 1 gm of molten PEG to make 50% (w/v) PEG and 12.5% (v/v) DMSO. Transfer the 50% PEG to a 37° water bath ready for use.

3. A. ii. Preparation of cells.

WI-L2-729-HF_2 tumor cells are harvested at $\cong 10^6$ cells per ml and washed twice in RPMI 1640. Lymphocytes after appropriate pre-culture treatments, are harvested, washed twice in RPMI 1640 and then counted.

3. A. iii. Fusion.

Using a 50 ml conical centrifuge tube, mix up to 2.5×10^7 lymphocytes and 2.5×10^7 WI-L2-729-HF_2 cells and pellet; the procedure can be scaled down to using as few as 10^6 of each cell type. To the pelleted cell mixture 2 ml of PEG/DMSO is added and gently swirled to get the cells into suspension. After 7 min at room temperature, over about 30 sec add 50 ml of RPMI 1640 and gently pellet the cells. Resuspend the pellet in 20 ml of R20F medium (or to about 2×10^6 total cells per ml) and incubate in 100 mm plastic tissue culture dishes overnight at 37° in an atmosphere of 5% CO_2 in air.

3. A. iv. Preparation of R20F medium.

To 90 ml of RPMI 1640 add:
1ml of 1M HEPES buffer.
1 ml of 100 mM sodium pyruvate.
1 ml of 100 × non-essential amino acids.
1 ml of Fungi-Bact (MA Bilogicals)
2 ml of 200 mM glutamine.
20 ml of FCS from pre-screened batches.
10 µl of 2-mercaptoethanol (0.5 M stock).

3. A. v. Plating of hybridomas for selection in HAT.

After overnight incubation, cells are plated at $1\text{-}2 \times 10^5$ cells per 0.1 ml in 96 well trays. Next day (24 hr post fusion) each well receives 0.1ml of 2 × HAT. One week later 0.l ml of medium is replaced with fresh 1 × HAT; thereafter, no exchange of media occurs until clones are macroscopically visible (3-5 weeks), at which time supernatants are harvested and the wells re-fed with 1 × HAT.

HAT is prepared from a 100 × HT supplement (supplied by Whittaker M.A. Bioproducts) and 100 × Aminopterin (Sigma) which is prepared by dissolving 1.76 mg of aminopterin in 100 ml of water.

3. A. vi. Stimulation of lymphocytes in culture.

3. A. vi. a. Fetal calf, Human A/B and serum-free media

Fetal calf serum is pre-screened to support a high cloning efficiency of WI-L2-729-HF_2 and hybridomas; lot-to-lot variation is considerable and usually about 1 in 10 is suitable. Several different suppliers have been tested with no overall superiority of any one.

Human AB serum pools are obtained from Whittaker M.A. Bioproducts.

Serum-free media; three versions have been used; HB101, Hanna Biologicals and Nutricyte, J. Brooks Laboratories, and a "home-grown" version of Iscove's medium developed in our laboratory by Dr. M. Gersten (ref. 5).

3. A. vi. b. Preparation of SAC (reformalinized Pansorbin).

As supplied by Calbiochem, Pansorbin is a 10% (w/v) suspension of heat inactivated and formalin fixed *S. aureus* (Cowan I strain) cells in PBS. To reduce the shedding of cell wall components, the reformalinization of Pansorbin has been recommended. This is accomplished by resuspending the pellet of a 5 ml aliquot of Pansorbin in 5 ml of 1.5% formaldehyde and incubating at room temperature for 1 hr. Cells are then washed twice in 50 ml of PBS and reconstituted to 5 ml (i.e., a 10% suspension).

3. A. vi. c. Lymphocyte culture conditions.

Lymphocytes (PBL or tonsil) are cultured in RPMI 1640 supplemented with 10% (v/v) serum, in serum-free medium alone, or serum-free medium supplemented with 1% to 2% serum at concentrations ranging from 2 to 3×10^6 per ml in plastic tissue culture dishes, tissue culture flasks, or in 24 well type tissue culture plates at 37°C with an atmosphere of 5% CO_2 in air. When appropriate, SAC, TCGF, 2-way MLR supernatant or soluble antigen was supplemented pre- or post-fusion. In the case of SAC a 0.01% to 0.025% final concentration was routinely used. Amounts of TCGF or 2-way MLR supernatants varied from 5% to 10% final concentrations. In experiments designed to further evaluate the effects of mononuclear phagocytes, adherent cells are removed by incubating cells on plastic tissue culture dishes or acid washed glass petri dishes in either a one-stage or two-stage adherence for various lengths of time at 37°C and removing the non-adherent cells by means of gentle trituration, followed by a wash.

As the recovery of non-adherent cells tended to be very poor utilizing acid washed glass petri dishes, even after only twenty minutes, this technique was discontinued. When supplies of lymphapheresis products (Hemacare or Green Hospital) began to consistently be provided with less than 30% mononuclear phagocytic cells adherence manipulations could be abandoned altogether. Erythrocytes are removed in the manner previously described, except that the blood or lymphapheresis packs are diluted with three volumes of saline before further processing.

3. A. vii. Screening of hybrids for immunoglobulin isotypes.

Plates of the 96 well type (Falcon #3911) are sensitized after removing static with a damp paper towel. A 1:200 dilution of goat anti-human kappa (supplied by TAGO or CALTAG), a 1:700 dilution of goat anti-human lambda (supplied by TAGO), or a 1:1000 dilution of goat anti-human IgM and goat anti-human IgG (supplied by CALTAG) in PBS-AE (phosphate buffered saline containing 0.0065% azide and 0.04% EDTA) is used to sensitize plates. The sensitization is carried out overnight at 4%C. Plates are then washed thoroughly with deionized water, followed by one wash with PBS-AE plus 0.05% tween. Until recently the plates were blocked by adding 0.1 ml of 1% (w/v) BSA (bovine serum albumin, supplied by SIGMA) in PBS-AE and were allowed to stand for at least 45 min at room temperature. As 0.1% gelatine (w/v) in PBS-AE gave significantly lower backgrounds, this is used routinely now. Plates are then washed twice with deionized water, followed by one wash with PBS-AE plus 0.05% tween.

To appropriately sensitized plates 45 μl of antibody (usually hybridoma culture supernatants) is added and plates allowed to stand overnight at 4°C. Next morning plates are warmed to room temperature, the supernatants drained, and plates washed twice with deionized water, followed by one wash with PBS–AE plus 0.05% tween, then allowed to drain.

After plates have bound antibodies present in the culture supernatants, 50 μl of appropriately diluted goat anti–human immunoglobulin that has been coupled with alkaline phosphatase is added and the plates allowed to incubate for 3–4 hours at 37°C. Plates are then washed twice with deionized water, then once with PBS–AE plus 0.05% tween and allowed to drain.

To determine the amount of human immunoglobulin bound to the plates 50 μl of nitrophenyl phosphate is added and the plates allowed to stand overnight at 4°C if the amount of human antibody bound to the plates is moderate, or allowed to stand overnight at room temperature if the amounts are low. The amount of nitrophenol released is measured photometrically using an automated plate reader (Dynatech) illuminated with 405 nm wavelength light.

The various goat anti–human immunoglobulins conjugated with alkaline phosphatase as purchased (TAGO or CALTAG) are diluted 1:20,000 in 1%BSA in PBS–AE. The nitrophenyl phosphate substrate is prepared as a 1 mg/ml solution in 0.05 M sodium bicarbonate buffer pH 8.0.

3. A. viii. Screening of hybridomas for Diphtheria Toxoid (DT) binding.

Essentially the same techniques as described for assaying immunoglobulins are used to assay the binding of hybridoma culture supernatants to Diphtheria Toxoid and Tetanus Toxoid from the Massachusetts Public Health Laboratories (1 μg/ml each) Ovalbumin from Sigma (1 μg/ml), Keyhole Limpet Hemocyanin from Calbiochem (0.1 μg/ml), BSA (1 μg/ml), HSA (human serum albumin from Sigma using a 1 μg/ml), and Dextran from National Biochemical Corporation, M.W. 5–40 million (100 μg/ml). The amounts used to typically sensitize plates were determined using normal human sera as a positive control.

3. A. ix. Human T–cell growth factor (TCGF) and 2–way mixed lymphocyte reaction (MLR) supernatants.

TCGF, a commercially available (Cellular Products, Inc.) tissue culture grade product that has been analyzed for its ability to stimulate proliferation of seven to ten day old, PHA transformed human T–cells. With such T–cells this preparation will induce a minimum five–fold increase in cell concentration when used at a final concentration of 10%.

The 2–way MLR supernatant was prepared by admixing 2×10^6 unfractionated PBL from each of two donors in 2 ml final volume complete media in 24 well tissue culture wells for 48 hours. Culture supernatants are then harvested, centrifuged, and filtered before use.

3. A. x. Selection of non–secreting WI–L2–729–HF_2 variants.

A non–secreting variant of WI–L2–729–HF_2 has been selected after multiple rounds of mutagenesis and by assaying clones for the absence of surface immunoglobulin. One clone, $62^{\times}60$ is stably κ–negative but μ chain expression is suppressed rather than strictly absent; i.e., hybrids formed with this clone and normal B–cells result in the re–expression of the μ–heavy chain. Our failure to recover a stable μ negative mutant after extensive frame–shift mutagenesis (over 300 clones have been analyzed) suggests that either there are two functional μ chain genes in WI–L2–729–HF_2, or that the tumorigenesis gene is intimately linked to μ chain gene presence and any structurally defective μ chain gene causes a loss of the tumor phenotype. While these possibilities are under study in our laboratory, we have at present no better line than $62^{\times}60$ representing a non–secreting variant of WI–L2–729–HF_2.

4. RESULTS

The results presented in this section represent a small fraction of the total experimental effort conducted under this contract. Since much of our data is negative in the sense that we have been unable to isolate a hybridoma secreting an antibody of defined specificity, much of the data has been omitted from this report. A broad cross-section of our results have been taken to illustrate some of the approaches and much of the inconsistency of the system. The results discussed in some detail focus on the fusion efficiencies we obtained because we have no antigen-specific hybrids to discuss. At the end of this section is a collection of various experiments and a data sheet showing some of the tests that were performed. Throughout the detailed results section reference will be made to specific experiment numbers which are also used to identify the data sheets given at the end of this section

4. A. Experiments using unfractionated PBL cultured under various conditions using serum-free media.

Iscove's medium and Nutricyte-H were tested in a variety of experiments with or without antigen, Diphtheria Toxoid (DT) and S. Aureus (Cowan Strain I) (SAC). Fusion efficiencies are given as frequencies $\times 10^6$, of hybrids relative to the number of PBL; results calculated in terms of total PBL and blasts at the time of fusion are presented in Table 1.

Table 1

Expt.	Donor	Medium	Days of Culture	SAC	DT	Fusion effic. $\times 10^6$
1	10	Iscove	7	+	–	0.7/0.2
4A	11	Iscove	7	+	+	0.8/4
36		Nut. H	4	–	–	0.4/2.7
					50 ng/ml	1.2/6.4
					5 ng/ml	1.6/10
					0.5 ng/ml	0.1/3.3
					0.05 ng/ml	0.4/nd
37		Nut. H	4	–	0.05 ng/ml	1.1/1.3
		Nut. H + 1% FCS		–	–	1.1/nd
					0.5 ng/ml	1.0
					1.0 ng/ml	0.2
39	20	Nut. H	5	–	–	0.04/0.07
					50 ng/ml	$<0.01/<0.02$
					100 ng/ml	1.4/2.4

Every attempt has been made to achieve consistency in the way PBL are isolated by lymphapheresis. However, the differential counts made on each preparation showed a wide variation in the numbers of mononuclear phagocytes. In order to reduce some of this variability we examined the effect of depleting cells which adhere to plastic or glass.

4.B. Experiment to test the effect of removing glass or plastic–adherent cells.

In one particularly informative experiment we divided PBL into three aliquots and used different media to carry–out the adherence. PBL were washed and resuspended in Nutricyte H (serum–free), RPMI supplemented with 5% fetal calf serum, or RPMI supplemented with 5% autologous serum. A few adherent cells had been removed; the remaining cells were adjusted to 3×10^6/ml and cultured for 7 days in the presence of no stimuli, 1 ng/ml DT, 1 ng/ml DT and 0.025% SAC, or 0.5 ng/ml DT and 0.025% SAC prior to fusion. The results summarized in Table 2 show that on the one hand removal of adherent cells in Nutricyte H is significantly better in terms of overall fusion efficiency, but on the other hand removal of adherent cells in RPM I supplemented with 5% autologous serum gave the best differential fusion efficiency of antigen–stimulated cultures relative to the SAC stimulated controls.

Table 2

Expt. No. 20

	fusion efficiencies $\times 10^6$ culture additions			
depletion media	SAC	1ng/ml DT	SAC + 1ng/ml DT	SAC + 0.5ng/ml DT
autologous plasma	0.14/0.4	0.57/1.3 (4.7/3.2)*	2.0/5.0 (14.3/12.5)	1.3/2.8 (9.3/7.0)
Fetal Bovine	2.0/5.6	4.2/11 (2.1/2.0)	0.8/0.7 (0.4/0.1)	0.67/1.3 (0.3/0.2)
Nutricyte H	7.0/13	6.3/11 (0.9/0.8)	18/40 (2.6/311)	30/60 (4.3/4.6)

* Values in parenthesis represents the increased yield of hybrids relative to groups receiving SAC alone.

In interpreting these results we were more impressed by the improved fusion efficiencies obtained with Nutricyte H than the relative increases in fusion efficiency obtained with autologous serum. We reasoned that our *in vitro* immunization procedures would make a marked difference in the relative numbers of antigen–specific B–cell blasts and with a more highly efficient way of capturing B–cells the none B–cell of interest might be more efficiently captured. The roughly 10–fold higher fusion efficiency outweighed in our judgment the approximately 10–fold higher selectivity found when adherent cells were removed in RPMI supplemented with autologous serum.

Subsequent experiments to test some of the *in vitro* immunization parameters were, therefore, carried out using non–adherent PBL obtained following adherence in Nutricyte H.

4. C. Experiments to test the effectiveness of lymphokines in improving the yield of antigen–specific hybridomas.

In this particular example we continued our comparison of non–adherent and unfractionated PBL and included one group in which unfractionated unstimulated cells were cultured for 4 days and after fusion 10 ng/ml DT was added initially and remained until diluted by feeding. The results are summarized in Table 3

Table 3

Expt. No. 37

	- MLR Supe	+ MLR Supe
unfractionated PBL		
+ 0.05 ng/ml DT	1.1/1.3	0.2/1.4 (5%)
		0.06/0.3 (10%)
unfractionated unstimulated PBL cultured 4 days		
+ 10ng/ml DT **after** fusion	6.6/55	
adherent cells removed in Nutricyte H		
+ 0.05 ng/ml DT	<0.04/<0.14	<0.02/<0.09

The results obtained in this experiment appear to contradict our findings presented in Table 2 in as much that the non-adherent cells obtained after removal of adherent cells in Nutricyte H were a poor substrate for hybridoma formation. However, this experiment used PBL from a different donor and cells were cultured for 4 rather than 7 days prior to fusion. Comparing groups which were cultured in either 5% or 10% MLR supernatant it would appear that lymphokines in an MLR supernatant are, if at all, inhibitory. The surprise in this experiment was that adding DT post-fusion resulted in an unusually high yield of hybrids.

The inhibitory effect of MLR supernatants was not unexpected given the findings of ourselves and others that crude PHA supernatants are inhibitory. The group which received antigen after fusion was included on the grounds that heterokaryon formed with resting B-cells and WI-L2-729-HF_2 do not normally grow, but upon interaction with antigen the B-cell heterokaryon may be activated and the immunoglobulin-secreting signals provided by the WI-L2-729-HF_2 may be significant in stabilizing the heterokaryon as a normal hybridoma. Judging from the results of this experiment our hunch appeared promising.

In a second series of experiments unfractionated PBL were cultured for 5 days in Nutricyte H in the presence or absence of 10% MLR supernatant as indicated in Table 4.

Table 4

Expt. No. 39

	fusion efficiency $\times 10^{-6}$	
Treatment	- Supe	+ Supe
No antigen	0.04/0.07	
+50ng/ml DT	<0.01/<0.02	0.2/0.8
+100ng/ml DT	1.4/2.4	4.7/15
+5ng/ml DT **after** fusion		0.16/0.07

At first sight the results of this experiment are in contradiction to those summarized in Table 3. However, the enhancing effect of a 10% (v/v) MLR supernatant seen in this experiment (c.f., inhibition in the previous experiment) may be a function of the 1–200–fold higher concentration of DT antigen. It is also interesting to note that while in Table 3 adding DT after fusion had a marked effect, in Table 4 where PBL were cultured in MLR supernatant and DT was added after fusion, the effect is almost non–existent.

One further experiment (Expt. No. 31) illustrates the effect of semi–purified T–cell growth factor to extend the proliferative response of B–cells. After removing adherent cells during culture for 1 hr on acid–washed glass plates in Nutricyte H the non–adherent fraction was cultured at 2×10^6/ml in Nutricyte H containing 10% (v/v) TCGF and 0.125 ng/ml DT for 5 days prior to fusion at a 1:1 ratio.

We obtained a low fusion efficiency of 3×10^{-8} based on total cell but with respect to blasts the fusion efficiency was 8×10^{-6}; i.e., over 100–fold higher. We interpret this to indicate that in the near absence of macrophages T–cells present in the PBL population do not proliferate well whereas the B–cells do and these form the majority of blasted cells.

4. D. Experiments to test the effects of "abnormal" help.

As we and others have shown previously, X–irradiated or mitomycin C–treated T–cells can serve as a source of "helper" activity that is antigen–non–specific. Three experiments illustrate our experience.

Before describing these experiments we might draw attention to the very low recoveries of cells after removing adherent cells in Nutricyte H. Since we are limited in the total numbers of PBL available from a single donor, when few non–adherent cells are recovered there is a necessary reduction in the number of treatment groups in any one experiment. Thus, a lack of "controls" is often the consequence of a poor recovery of non–adherent cells.

Three experiments testing "abnormal" help, non–adherent PBL resulting from the removal of adherent cells in either Nutricyte H or RPMI supplemented with autologous plasma. Several variables were examined, including time of culture (3 or 6 days) the ratio of mitomycin C–treated allogeneic PBL relative to the non–adherent responding PBL, and the antigenic stimulus (DT ± SAC).The results of these experiments are summarized below.

In the first experiment in this series adherent cells were removed in Nutricyte H for 45 minutes (in general adherence was for 24 hours) and there were 1/5 as many mitomycin C–treated "helpers" relative to the number of responding PBL (Expt. No. 22A). The results of this preliminary

experiment were beyond expectations yielding $>18\times10^{-6}$ and $>77\times10^{-6}$ fusions efficiencies based on total cells and blasts respectively.

The second experiment used non–adherent cells recovered after adherence in Nutricyte H were cultured for 4 days in the presence of DT and mitomycin C–treated allogeneic PBL. Only a single treatment group was scored.

Table 5

Expt. No. 29

	fusion efficiencies $\times10^6$ days of culture	
Culture conditions	3	6
(i) Adherent cells removed in Nutricyte H		
+ 1ng/ml DT + 0.45 allo "helpers"	1.3/–	/–
+ 1ng/ml DT + 0.09 allo "helpers"	0.4/–	/–
(ii) Adherent cells removed in RPMI supplemented with autologous plasma		
+ 1ng/ml DT + 0.45 allo "helpers"	7.4/–	4.1/–
+ SAC + 1ng/ml DT + 0.45 allo "helpers"	/–	1.5/–
+ SAC + 1ng/ml DT + 0.07 allo "helpers"	13/–	1.0/–

Note: Blasts were not scored in this experiment.

In the third experiment (Expt. No. 30) adherent cells removed in Nutricyte H and cultured for 4 days in 0.5 ng/ml DT and 1/2 the number of mitomycin C–treated allogeneic PBL ("helpers"). The fusion efficiencies were 2.9×10^{-6} based on total viable cells or 41×10^{-6} based on the blasts present at the time of fusion.

Although there is a clear tendency in this experiment for higher fusion efficiencies in groups where non–adherent cells were removed in the presence of autologous plasma (in contrast to results summarized in Table 2), it is possible that the impaired fusion efficiencies were generally related to the stronger effect of the allogeneic "abnormal" helpers. In fact it would appear that the day 3 cultures were more efficient at hybridoma formation when a small number of "helpers" (a ratio of 0.07) was added along with SAC and DT, as compared with a ratio of 0.45.

4. E. Side–by–side comparison of different donors.

PBL were depleted of adherent cells by incubation in RPMI/FCS for 24 hr in plastic dishes. Non–adherent cells were cultured for 1.5 days in Nutricyte H before adding SAC and DT before continuing the culture for a total of 7 days. Cells were fused in a ratio of 1:1 with WI–L2–729–HF_2. The results are summarized in Table 7 below.

Table 7

Expt. No. 6A

culture additions	donor 11	12
+ SAC + 1 ng/ml DT	8.1/31	0.33/2.0
+ SAC + 10 ng/ml DT	0.88/4.0	0.03/0.2
+ SAC + 100 ng/ml DT	2.8/4.0	1.2/9.0
+ SAC + 1 μg/ml DT	1.0/4.0	<0.03
- SAC + 10 ng/ml DT	2.5/11	<0.02

Adherent cells were removed by 24 hr incubation in Nutricyte H on plastic. Non-adherent cells were cultured for 7 days before fusion at a ration of 1:1 with WI-L2-729-HF_2. The results are summarized in Table 8.

Table 8

Expt No. 22

culture additions	donor 13	14
+ SAC + 1 ng/ml DT	1.3/2.0	<0.59/<0.17
+ SAC + 500 pg/ml DT	0.67/0.88	<0.77/<0.17
+ SAC + 125 pg/ml DT	1.5/2.4	1.1/3.3
+ SAC + 62.5 pg/ml DT	0.91/1.5	2.9/6.7
+ SAC + 31 pg/ml DT	2.1/2.6	<2.0/<5.0

The results obtained from these two experiments amply illustrate the major differences that can occur when two different donors are compared under identical culture and fusion conditions. This finding indicates that a given donor, and possibly a single pool of PBL from a given donor, the optimal culture, stimulation and hybridization conditions will have to be determined before attempting to initiate an *in vitro* immunization protocol.

4. F. The effect of cell density during pre-culture.

Although earlier experiments had shown that the optimal concentration of cells during pre-culture to be 2×10^6/ml, we inadvertently set up parallel cultures for 4 days at 2×10^6/ml and 3×10^6/ml. The results are summarized in Table 9 below.

Table 9

Expt. No. 36

PBL cultured at additions to cultures	2×10^6	3×10^6
none	0.4/2.7	17/83
50 ng/ml DT	1.2/6.4	6.7/31
5 ng/ml DT	1.6/10	2.0/9.3
0.5 ng/ml DT	0.5/3.3	1.6/4.1
0.05 ng/ml DT	0.4/nd	0.2/nd

As a consequence of these findings we routinely set up all subsequent pre–cultures at 3×10^6 cells per ml. Although not every donor gave rise to hybridomas at the high frequencies found in this case, as a general rule fusion efficiencies were higher and of all the apparent anti–DT hybridomas we isolated were obtained from experiments in which the higher cell density was used during pre–culture.

4. G. The effect of pre–culture period.

In this experiment we examined the effect of pre–culture time on the generation of hybridomas under conditions where unfractionated PBL were incubated for 1 to 4 days at 3×10^6/ml in Nutricyte H in the presence or absence of DT antigen. The results are summarized in Table 10 below.

Table 10

Expt. No. 48

	days of pre–culture			
additions	1	2	3	4
none	0.62/5.0	1.2/3.6	<0.13/<0.20	<0.13/nd
50 ng/ml DT	1.0/2.8	<0.02/<0.2	<0.17/<0.5	<0.17/nd
10 ng/ml DT	>30/>80	0.5/1.3	1.0/3.0	<0.17/nd
5 ng/ml DT	6.7/16	0.08/0.2	<0.11/<0.29	<0.13/nd
1 ng/ml DT	6.0/30	0.25/0.6	<0.14/<0.33	<0.13/nd
0.5 ng/ml DT	<0.05/<0.15	0.33/0.75	<0.17/<0.33	<0.13/nd
none (10 ng/ml DT **after** fusion)	0.20/0.80	5.1/14	0.33/0.67	<0.20/nd

Comparing the results obtained from this experiment an the previous one summarized in Table 9 it seems clear that while on day 4 the two are indicating vastly different results for a given treatment, the different donors in each case indicate that the optimum time to carry out fusions varies dramatically. We find the overall patterns of response to antigen, pre–culture period and fusion conditions to be sufficiently variable that little is to be gained by pursuing this problem further in the future.

4. H. An analysis of hybridoma clones showing apparent anti-DT activity.

Several hybridomas with anti-DT activity have been isolated. The closely studied group of 7 clones were derived from 3 independent fusions. In each case hybridoma supernatants were found to bind DT coated plates and they could be revealed with either a class-specific anti-human IgM or anti-human kappa. However, during the expansion and subsequent sub-cloning steps it became apparent that detection of anti-DT activity correlated with λ light chain and γ heavy chain expression, yet neither of these two chains could be found bound to DT-coated plates. Even more puzzling was our finding that anti-DT binding could appear and disappear in the culture supernatants within 2-4 days. After repeated sub-cloning we were able to isolate 2 stable DT-binding clones which did not exhibit transient expression.

Upon further testing of these putative anti-DT clones we found that they were all quite non-specific and tended to bind any negatively charged surface, including the protein-blocked ELISA plates. We have no explanation for this pattern of behavior, although we did test and eliminate, the possibility that the IgM was anti-degraded and aggregated IgG which was non-specifically "sticky".

At this stage we felt justified in putting aside our studies on human hybridomas *per se*. We will now investigate the *in vitro* immunization protocols more intensively in an effort to establish a working immunization scheme. However, in view of the unexpected difficulty in isolating a completely IgM-negative mutant, there may be good reason to resume work with the hybridomas when this problem has been resolved.

4. I. DETAILED PRESENTATION OF DATA

Date: 1/9/87 Expt. No. 1

Donor No. 10 Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

A Whole population set up at 2×10^6/ml in Iscove's + 10 μg/ml human transferrin + 10 μg/ml bovine insulin + 0.01% SAC; fuse day 7 1:1 PBL to tumor.

B Whole population set up at 2×10^6/ml in Iscove's + 10 μg/ml human transferrin + 10 μg/ml bovine insulin for 2.5 days; then add 0.01% SAC; fuse day 7 1:1 PBL to tumor.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	6.6×10^{-7}	1.2×10^{-6}	23	23	13	12	nd	nd	0
B	7.7×10^{-8}	1.5×10^{-7}	2	2	0	2	nd	nd	0

Date: 1/14/87 Expt. No. 4A

Donor No. 11 Cell Type: PBL Tumor or Parent: WI-L2-HF_2

Pre–Treatment of Cells

A Whole population set up at 2×10^6/ml in Iscove's + 10 μg/ml human transferrin + 10 μg/ml bovine insulin + 0.01% SAC + 5ng/ml DT; fuse 1:1 (day 7) PBL to tumor.

B Whole population set up at 2×10^6/ml in Iscove's + 10 μg/ml human transferrin + 10 μg/ml bovine insulin for 2.5 days, then add 0.01% SAC + 5ng/ml DT; fuse day 7 1:1 PBL to tumor.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	8×10^{-7}	4×10^{-6}	50	48	18	13	nd	nd	0
B	7×10^{-8}	2×10^{-7}	4	4	0	3	nd	nd	0

Date: 1/21/87 Expt. No. 6A

Donor No. 11 & 12/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

A 24 hour adherence (plastic) in RPMI + 10% FCS; non-adherent into Nutricyte H alone 1.5 days; then add 0.01% SAC and 1ng/ml DT for 4.5 days; fuse day 7 1:1 PBL to tumor. Donor 11.

B Same as A, but 10ng/ml DT.

C Same as A, but 100ng/ml DT.

D Same as A, but 1 μg/ml DT.

E 24 hour adherence (plastic) in RPMI + 10% FCS; non-adherent into Nutricyte H alone 1.5 days; then add 10ng/ml DT for 4.5 days; fuse day 7 1:1 PBL to tumor.

F Same as A, except Donor 12.

G Same as B, except Donor 12.

H Same as C, except Donor 12.

I Same as D, except Donor 12.

J Same as E, except Donor 12.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	8.1×10^{-6}	3.1×10^{-5}	47	47	20	20	nd	nd	0
B	8.8×10^{-7}	4.0×10^{-6}	8	8	5	3	nd	nd	0
C	8.0×10^{-7}	2.8×10^{-6}	8	8	6	2	nd	nd	0
D	1.0×10^{-6}	4.0×10^{-6}	11	11	7	2	nd	nd	0
E	2.5×10^{-6}	1.1×10^{-5}	11	11	6	2	nd	nd	0
F	3.3×10^{-7}	2.0×10^{-6}	2	2	2	0	nd	nd	0
G	$<2.5\times10^{-8}$	$<2\times10^{-7}$							
H	1.2×10^{-6}	9.0×10^{-6}	5	5	2	3	nd	nd	0
I	$<3.0\times10^{-8}$	nd							
J	$<2.4\times10^{-8}$	nd							

Date: 2/2/87 Expt. No. 11

Donor No. D/Fresh Cell Type: Tonsil Tumor Parent: WI-L2-HF_2

Pre–Treatment of Cells

A Whole population at 2×10^6/ml in Iscove's + 10 μg/ml human transferrin and 10 μg/ml bovine insulin + 0.01% SAC; fuse day 4; plate out at 2×10^4/well.

B Whole population at 2×10^6/ml in Iscove's + 10 μg/ml human transferrin and 10 μg/ml bovine insulin + 0.01% SAC and 1 μg/ml DT; fuse day 4; plate out at 2×10^4/well.

C Same as A, except plate out at 1×10^4/well.

D Same as B, except plate out at 1×10^4/well.

	Fusion efficiency:				wellsIg in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	3.0×10^{-6}	2.3×10^{-5}	6	6	4	2	nd	nd	0
B	9.0×10^{-6}	3.3×10^{-5}	18	18	13	5	nd	nd	0
C	4.2×10^{-5}	3.2×10^{-4}	12	12	10	2	nd	nd	0
D	4.8×10^{-6}	1.8×10^{-5}	12	12	10	2	nd	nd	0

Date: 2/11/87 Expt. No. 12

Donor No. F/Frozen Cell Type: Tonsil Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

A Direct Fusion (No Stimulation) – fused 1:2 tonsil to tumor. Plate out at 6.6×10^4/well.

B Direct Fusion (No Stimulation) – fused 1:5 tonsil to tumor. Plate out at 6.6×10^4/well.

C Direct Fusion (No Stimulation) – fused 1:2 tonsil to tumor. Plate out at 3.3×10^4/well.

D Direct Fusion (No Stimulation) – fused 1:5 tonsil to tumor. Plate out at 3.3×10^4/well.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	4.0×10^{-6}	nd	20	18	7	8	nd	nd	0
B	$>8.0 \times 10^{-5}$	nd	>400	248	45	203	nd	nd	0
C	2.4×10^{-6}	nd	11	8	5	2	nd	nd	0
D	$>1.4 \times 10^{-4}$	nd	>700	0					

Date: 2/20/87 Exp. No. 15

Donor No. F & I/Frozen Cell Type: Tonsil Tumor Parent: WI-L2-HF_2

Pre–Treatment of Cells

A Tonsil F: set up at 2×10^6/ml in 60 x 15 mm T.C. dish (5 ml/dish) and 10 ng/ml DT and 0.01% SAC added; fused day 4 1:5 tonsil to tumor.

B Tonsil I: set up at 2×10^6/ml in 60 x 15mm T.C. dish (5 ml/dish) and 10 ng/ml DT and 0.01% SAC added; fused day 4 1:5 tonsil to tumor.

	Fusion Efficiency:		wells		Ig in Supernatants				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	1.4×10^{-5}	1.8×10^{-4}	7	7	4	2	nd	nd	0
B	5.7×10^{-6}	no blasts	2	2	0	2	nd	nd	0

Date: 2/26/87 Expt. No. 17

Donor No. 13/Fresh Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre–Treatment of Cells

A 24 hour adherence (plastic) in Nutricyte H; add 0.01% SAC to non–adherent population (day 1), fuse day 7 1:2 PBL to tumor.

B Same as A, except fuse 1:5 PBL to tumor.

C 24 hour adherence (plastic) in Nutricyte H, add 0.01% SAC and 1 μg/ml DT to non–adherent population (day 1), fuse day 7 1:2 PBL to tumor.

D Same as C, except fuse 1:5 ratio PBL to tumor.

E 24 hour adherence (plastic) in Nutricyte H, add 0.01% SAC to non–adherent population (day 1); add 1 μg/ml DT on day 2; fuse day 7 1:2 PBL to tumor.

F Same as A, except fuse day 8.

G Same as C, except fuse day 8.

H Same as E, except fuse day 8.

I Same as E, except fuse day 8 1:5 PBL to tumor.

Comments on hybrids: not very stable after 5–6 weeks in culture. Started dying off for some unknown reason.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	6.8×10^{-6}	1.2×10^{-5}	41	41	15	13	nd	nd	0
B	1.0×10^{-5}	1.8×10^{-5}	62	57	36	14	nd	nd	0
C	1.3×10^{-5}	2.0×10^{-5}	80	64	18	42	nd	nd	0
D	1.8×10^{-5}	3.2×10^{-5}	110	108	63	45	nd	nd	0
E	2.3×10^{-6}	1.2×10^{-5}	40	28	19	9	nd	nd	0
F	1.2×10^{-5}	2.2×10^{-5}	70	0					
G	1.3×10^{-6}	1.9×10^{-6}	12	0					
H	$<4.5\times10^{-8}$	$<9.1\times10^{-8}$	0	0					
I	$<4.5\times10^{-8}$	$<9.1\times10^{-8}$	0	0					

Date: 3/2/87 Expt. No. 20

Donor No. 13/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre-treatment of cells

A 48 hour*adherence (plastic) in 5% autologous plasma; add 0.025% SAC to non-adherent population; day 7-fuse 1:1 with WI-L2-HF_2.

B Same as A, except 48 hour adherence in RPMI + 10% FCS.

C Same as A, except 48 hour adherence in Nutricyte H.

D 48 hour adherence (plastic) in 5% autologous plasma; add 1 ng/ml DT to non-adherent population; day 7 fuse 1:1 with WI-L2-HF_2.

E Same as D, except 48 hour adherence in RPMI + 10% FCS.

F Same as D, except 48 hour adherence in Nutricyte H.

G 48 hour adherence (plastic) in 5% autologous plasma; add 0.025% SAC +1 ng/ml DT to non-adherent population; day 7 fuse 1:1 with WI-L2-HF_2.

H Same as G, except 48 hour adherence in RPMI + 10% FCS.

I Same as G, except 48 hour adherence in Nutricyte H.

J 48 hour adherence (plastic in 5% autologous plasma; add 0.025% SAC + 0.5 ng/ml DT to non-adherent population; day 7 fuse 1:1 with WI-L2-HF_2.

K Same as J, except 48 hour adherence in RPMI + 10% FCS.

L Same as J, except 48 hour adherence in Nutricyte H.

	Fusion Efficiency:		wells		Ig in Supernatants				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	1.4×10^{-7}	4.0×19^{-7}	1	1	0	1	nd	nd	nd
B	2.0×10^{-6}	5.6×10^{-6}	9	0			nd	nd	nd
C	7.0×10^{-6}	1.3×10^{-5}	35	0			nd	nd	nd
D	5.7×10^{-7}	1.3×10^{-6}	4	4	2	2	nd	nd	nd
E	4.2×10^{-6}	1.1×10^{-5}	15	0			nd	nd	0
F	6.3×10^{-6}	1.1×10^{-5}	15	0			nd	nd	0
G	2.0×10^{-6}	5.0×10^{-6}	9	8	3	5	nd	nd	0
H	8.0×10^{-7}	0.7×10^{-6}	4	0			nd	nd	0
I	1.8×10^{-5}	4.0×10^{-5}	55	0			nd	nd	0
J	1.3×10^{-6}	2.8×10^{-6}	5	4	0	4	nd	nd	0
K	6.7×10^{-7}	1.3×10^{-6}	4	0			nd	nd	0
L	3.0×10^{-6}	6.0×10^{-5}	71	0			nd	nd	0

Comments: *Adherences done in 2 (24 hour) stages; after 24 hours adherence, non-adherent were transferred to new dish for 24 hours further adherence. Note that those groups adhered in Nutricyte H have better efficiencies. This serum free formulation is conducive to the adherence of Mϕ's in a way not seen with just FCS, but recoveries at non-adherent very poor. Although fusion efficiencies were noted for several groups, the wells scored shows zero in those cases where clones were too unstable to work with

Date: 3/18/87 Expt. No. 22

Donor No. 13 & 14/frozen Cell Type: PBL Tumor Parent: Wl-L2-HF_2

Pre-Treatment of Cells

A Plastic adherence (24 hours) in Nutricyte H; set up non-adherent population in Nutricyte H + 1 ng/ml DT + 0.025% SAC; fuse 1:1 PBL to tumor day 7 - Donor #13.

B Same as A, except 500 pg/ml DT.

C Same as A, except 125 pg/ml DT.

D Same as A, except 62.5 pg/ml DT.

E Same as A, except 31.25 pg/ml DT.

F Same as A, except Donor #14.

G Same as B, except Donor #14.

H Same as C, except Donor #14.

I Same as D, except Donor #14.

J Same as E, except Donor #14.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	1.3×10^{-6}	2.0×10^{-6}	4	4	3	0	nd	nd	0
B	6.7×10^{-7}	8.8×10^{-7}	3	3	2	0	nd	nd	0
C	1.5×10^{-6}	2.4×10^{-6}	5	5	1	1	nd	nd	0
D	9.1×10^{-7}	1.5×10^{-6}	3	3	1	0	nd	nd	0
E	2.1×10^{-6}	2.6×10^{-6}	9	9	2	2	nd	nd	0
F	$<5.9\times10^{-7}$	$<1.7\times10^{-7}$							
G	$<7.7\times10^{-7}$	$<1.7\times10^{-7}$							
H	1.1×10^{-6}	3.3×10^{-6}	1	1	0	0	nd	nd	0
I	2.9×10^{-6}	6.7×10^{-6}	2	2	1	0	nd	nd	0
J	$<2.0\times10^{-6}$	$<5.0\times10^{-6}$							

Date: 4/8/87 Expt. No. 22A

Donor No. 11 & 14/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

A Macrophage depletion on acid washed glass petri dishes for 45 minutes in Nutricyte H (each donor); non-adherent #11 mitomycin C treated with 50 μg/ml; admix non-adherent #14 and non-adherent mitomycin C treated #11 in Nutricyte H at 3×10^6/ml with 1/5 being mitomycin C treated #11 cells; fuse day 5 1:1 PBL to tumor.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	$>1.8 \times 10^{-5}$	$>7.7 \times 10^{-5}$		88					

Comments: Day 3 supes from pre-treatment were DT negative, κ positive, λ positive and IgG positive. 100% of wells had hybrids, obviously not clonal. No κ λ values determined, but of 88 tested, none DT positive, and 22 were IgG secretors.

Date: 4/16/87 Expt. No. 29

Donor No. 16/fresh, 13/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

A Donor #16 (fresh) *24 hour adhered at 2×10^6/ml in Nutricyte H; non-adherent population admixed with 0.45 × as many mitomycin C treated only donor #13 (frozen) in Nutricyte H at 2×10^6/ml + 1ng/ml DT; fuse day 3 1:1 PBL to tumor.

B Same as A, except fused day 6.

C Donor #16 (fresh) *24 hour adhered at 2×10^6/ml in Nutricyte H; non-adherent population admixed with 0.09 × as many Mitomycin C treated only donor #13 (frozen) in Nutricyte H at 2×10^6/ml + 1 ng/ml DT; fuse day 3 1:1 PBL to tumor.

D Same as C, except fused day 6.

E Donor #16 (fresh) *24 hour adhered at 2×10^6/ml in 5% autologous plasma; non-adherent population admixed with 0.45 × as many Mitomycin C treated only donor #13 (frozen) in Nutricyte H at 2×10^6/ml + 1 ng/ml DT; fuse day 3 1:1 PBL to tumor.

F Same as E, except fused day 6.

G Donor #16 (fresh) *24 hour adhered at 2×10^6/ml in 5% autologous plasma; non-adhered population admixed with 0.07 × as many Mitomycin C treated only donor #13 (frozen) in Nutricyte H at 2×10^6/ml + 1 ng/ml 01 + 0.01% SAC; fuse day 3 1:1 PBL to tumor.

H Same as G, except fused day 6.

I Donor #16 (fresh) *24 hour adhered at 2×10^6/ml in 5% autologous plasma; non-adhered population admixed with 0.45 × as many Mitomycin C treated only donor #13 (frozen) in Nutricyte H at 2×10^6/ml + 1 ng/ml DT + 0.01% SAC; fused day 3 1:1 PBL to tumor.

J Same as I, except fused day 6.

Comments: day 3 supes from culture were tested DT negative, kappa positive, lambda positive and IgG positive - on all groups.

*Note: 24 hour adherences in acid washed glass petri dishes.

RESULTS ON NEXT PAGE

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	1.3×10^{-6}	nd	2	1	0	0	nd	0	0
B	$<1.5\times10^{-6}$	nd	0						
C	4.0×10^{-7}	nd	1	1	0	0	nd	nd	0
D	$<2.8\times10^{-6}$	nd	0	0					
E	7.4×10^{-6}	nd	35	24	4	0	nd	1	0
F	4.1×10^{-6}	nd	14	11	5	1	nd	1	0
G	1.3×10^{-5}	nd	47	31	13	3	nd	5	0
H	1.0×10^{-6}	nd	11	4	2	0	nd	nd	0
I	$<6.7\times10^{-7}$	0	0						
J	1.5×10^{-6}	nd	7	4	0	0	nd	0	0

Date: 4/27/87 Expt. No. 30

Donor No. 17& 13/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2 OR 62 × 60

Pre-Treatment of Cells

A Plastic adherence In Nutricyte H for 1 hour (both donors); Mitomycin C treat donor #13 at 50 μg/ml. Admix both populations (donor #17 is non-adherent population - donor #13 is non-adherent, Mitomycin C treated, and 1/2 the number of donor #17 cells) in Nutricyte H + 0.5 ng/ml DT; fuse day 4 1:1 PBL to tumor.

B Same as A, except fuse with 62 × 60.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	2.9×10^{-6}	4.1×10^{-5}	23	15	4	3	14	1	0
B	$<1.3\times10^{-7}$	$<1.8\times10^{-6}$	0	0					

Date: 4/29/87 Expt. No. 31

Donor No. 15/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre–Treatment of Cells

A Whole population set up in Nutricyte H at 4×10^6/ml for 1 hr; non–adherent recovered and set up at 2×10^6/ml in Nutricyte H + 0.125 ng/ml DT + 10% TCGF (T–cell growth factor); fused 1:1 day 5 with WI-L2-HF_2.

Note: TCGF from Cellular Products, Inc.

	Fusion Efficiency:		wells		Ig in Supernatants				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	3×10^{-8}	8×10^{-6}	1	0					

Date: 5/4/87 Expt. No. 34

Donor No. 17/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2 OR 62^X60

Pre-Treatment of Cells

A plastic adhered 1 hr. at 4×10^6/ml in 10% autologous plasma; set up non-adherent at 3×10^6/ml in Nutricyte H + 0.05 ng/ml DT. Fused day 4 1:1 with WI-L2-HF_2.

B same as A, except fused 1:1 with 62^X60.

C adhered on acid washed glass petri dishes at 4×10^6/ml in 10% autologous plasma; set up non-adherent at 3×10^6/ml in Nutricyte-H + 0.05 ng/ml DT. Fused day 4 1:1 with WI-L2-HF_2.

D Same as C, except fused 1:1 with 62^X60.

E Whole population fused 1:1 (directly) with WI-L2-HF_2 (no pre-culture).

F Same as E, except fused 1:1 with 62^X60.

	Fusion Efficiency:		wells		Ig in Supernatants				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	1.4×10^{-6}	2.4×10^{-6}	10	0					
B	$< 4.0 \times 10^{-7}$	$< 2.4 \times 10^{-7}$	0	0					
C	1.4×10^{-6}	2.4×10^{-6}	0						
D	8.6×10^{-6}	1.5×10^{-6}	6	0					
E	$< 6.7 \times 10^{-8}$	nd	0	0					
F	$< 6.7 \times 10^{-8}$	nd	0	0					

Comments: None DT^+, IgG and IgM secretion not known.

Date: 5/7/87 Expt. No. 36

Donor No. 19 Cell Type: PBL Tumor Parent: WI-L2-HF_2 or OR 62 $\times$ 60

Pre–Treatment of Cells

cultured at 2 or 3×10^6/ml

A Whole population at 2×10^6/ml in Nutricyte H; fused 1:1 day 4 PBL to tumor-WI-L2-HF_2.

B Same as A, except fuse with 62 $\times$ 60

C Whole population at 2×10^6/ml in Nutricyte H + 50ng/ml DT; fuse day 4 1:1 PBL to tumor - WI-L2-HF_2.

D Same as C, except fuse with 62 $\times$ 60.

E Same as C, except 5 ng/ml DT.

F Same as C, except 0.5 ng/ml DT.

G Same as C, except 0.5 ng/ml DT and Fused day 4 1:1 PBL to tumor–62 $\times$ 60.

H Same as C, except 0.05ng/ml DT.

I Same as C, except 0.05 ng/ml DT and fused day 4 1:1 PBL to tumor–62 $\times$ 60.

RESULTS ON NEXT PAGE

Fusion efficiency: @ 2×10^6/ml wells					Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	4.0×10^{-7}	2.7×10^{-6}	4	0	nd	nd	nd	nd	0
B	$< 1.0 \times 10^{-7}$	$< 6.7 \times 10^{-6}$	0	0					
C	1.2×10^{-6}	6.4×10^{-6}	16	16	4	3	nd	1	0
D	1.9×10^{-7}	8.0×10^{-7}	2	2	0	2	nd	0	0
E	1.6×10^{-6}	1.0×10^{-5}	54	54	16	19	nd	2	0
F	5.0×10^{-7}	3.3×10^{-6}	8	8	2	4	nd	0	0
G	2.0×10^{-7}	1.7×10^{-6}	4	4	1	1	nd	0	0
H	4.4×10^{-7}	nd	5	5	3	1	nd	0	0
I	9.4×10^{-7}	nd	13	13	5	1	nd	0	0

Fusion efficiency: @ 3×10^6/ml wells					Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	1.7×10^{-5}	8.3×10^{-5}	500	500	260	116	14*	32*	14
B	3.9×10^{-6}	1.9×19^{-5}	115	115	47	41	nd	15	0
C	6.7×10^{-6}	3.1×10^{-5}	138	138	85	39	4**	12**	4
D	$< 4.3 \times 10^{-8}$	$< 2.2 \times 10^{-7}$	0	0					
E	2.0×10^{-6}	9.3×10^{-6}	139	139	62	42	nd	6	0
F	1.6×10^{-6}	4.1×10^{-6}	31	31	13	7	nd	4	0
G	5.0×10^{-8}	4.0×10^{-7}	3	3	1	0	nd	1	0
H	2.0×10^{-7}	nd	4	4	3	1	nd	0	0
I	4.5×10^{-7}	nd	8	8	2	3	nd	1	0

Notes: *14 of these 32 were DT $^+$.

Same 14 were making new IgM-only DT $^+$ screened on IgM.

14 DT $^+$ were IgM specific.

**4 of IgG $^+$ were DT $^+$ and IgM specific.

(only 4 DT $^+$ clones screened on IgM coated plates.)

Date: 5/18/87 Expt. No. 37

Donor No. 18/frozen Cell Type: PBL Tumor Parent: WI-L2-HF_2

A Donor cells adhered 1/2 hour on acid washed glass petri dishes in Nutricyte H; non-adhered setup at 3×10^6/ml in Nutricyte H + 0.05 ng/ml DT + 5% 2-way MLR supe; fused day 4 1:1 PBL to tumor.

B Same as A, except no 2-way MLR supe.

C Whole population cells setup at 3×10^6/ml in Nutricyte H alone; fused day 4 1:1 PBL to tumor with 10 ng/ml DT added post fusion.

D Whole population cells setup at 3×10^6/ml in Nutricyte H + 0.05 ng/ml DT; fused day 4 1:1 PBL to tumor.

E Whole population cells setup at 3×10^6/ml in Nutricyte H + 0.05 ng/ml DT + 5% 2-way MLR supe; fused day 4 1:1 PBL to tumor.

F Same as E, except 10% 2-way MLR supe.

Comments: Day 4 supes from pretreatment show all groups secrete kappa, lambda and IgG. No DT activity.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	$<2.0 \times 10^{-8}$	$<9.1 \times 10^{-8}$	0	0					
B	$<3.6 \times 10^{-8}$	$<1.4 \times 10^{-7}$	0	0					
C	6.6×10^{-6}	5.5×10^{-5}	66	65	24	27	4*	7*	4*
D	1.1×10^{-6}	1.3×10^{-6}	12	12	7	5	nd	1	0
E	2.2×10^{-7}	1.4×10^{-6}	5	5	2	2	nd	0	0
F	5.9×10^{-8}	2.8×10^{-7}	1	1	0	1	nd	0	0

*Note: 4 of 7 IgG^+ were DT^+ and IgM specific (only those 4 clones tested on IgM coated plates).

Date: 5/21 87 Expt. No. 39

Donor No. 20/fresh Cell type: PBL Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

A Whole population set up at 3×10^6/ml in Nutricyte H + 10% 2-way MLR (Mixed lymphocyte reaction) supe; fuse day 5 1:1 PBL to tumor—add 5 ng/ml DT post-fusion.

B Same as A, except no MLR supe and no DT post-fusion.

C Whole population set up at 3×10^6/ml in Nutricyte H + 50 ng/ml DT; fuse day 5 1:1 PBL to tumor.

D Same as C, except 100ng/ml DT.

E Whole population set up at 3×10^6/ml in Nutricyte H + 50 ng/ml DT and 10% MLR supe; fuse day 5 1:1 PBL to tumor.

F Same as E, except 100 ng/ml DT.

	Fusion efficiency:		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT
A	1.6×10^{-7}	4.6×10^{-7}	11	10	5	3	nd	nd	0
B	4.1×10^{-8}	6.9×10^{-8}	4	3	3	1	nd	nd	0
C	$< 1.2 \times 10^{-8}$	$< 2.0 \times 10^{-8}$	0	0					
D	1.4×10^{-6}	2.4×10^{-6}	*						
E	2.3×10^{-7}	7.7×10^{-7}	*						
F	4.7×10^{-6}	1.5×10^{-5}	*						

*Note: numbers are based on the approximate number of clones emerging in each group before being lost to mold.

Date. 8/24/87 Expt. No. 40A

Donor No. 18 Cell Type: PBL Tumor Parent: WI-L2-HF$_2$

Pre-Treatment of Cells: Fusions carried out days 1–4 of culture.

A Whole population cells set up at 3×10^6/ml in Nutricyte H + 1% FCS alone; fused 1:1 PBL to Tumor.

B Whole population cells set up at 3×10^6/ml in Nutricyte H + 1% FCS; fused 1:1 PBL to Tumor; 10 ng/ml DT added post fusion.

C Whole population ccells set up at 3×10^6/ml in Nutricyte H + 1% FCS + 0.5 ng/ml DT; fused 1:1 PBL to tumor.

D Same as C, except 1 ng/ml DT

E Same as C, except 5 ng/ml DT

F Same as C, except 10 ng/ml DT

G Same as C, except 50 ng/ml DT

	Fusion efficiency: Day 3		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	1.1×10^{-6}	nd	20	14	13	7	6	0	0
B	3.8×10^{-6}	nd	80	68	68	6	60	15*	0
C	1.0×10^{-6}	nd	20	4	1	0	0	0	0
D	2.0×10^{-7}	nd	4	2	2	2	0	0	0

* These same 15 cloes secrete IgM.

All but the data above was lost due to contamination.

Date: 9/7/87 Expt. No. 48

Donor No. 24 Cell Type: PBL Tumor Parent: WI-L2-HF_2

Pre-Treatment of Cells

Fusions carried out on days 1 – 4 of culture

A Whole population cells setup at 3×10^6/ml in Nutricyte H alone; fused 1:1 PBL to tumor.

B Whole population cells setup at 3×10^6/ml in Nutricyte H alone; fused 1:1 PBL to tumor; 10 ng/ml DT added post fusion.

C Whole population cells set up at 3×10^6/ml in Nutricyte H + 0.5 ng/ml DT; fused 1:1 PBL to tumor.

D Same as C, except 1 ng/ml DT.

E Same as C, except 5 ng/ml DT.

F Same as C, except 10 ng/ml DT.

G Same as C, except 50 ng/ml DT.

RESULTS ON NEXT PAGE

	Fusion efficiency: Day 1		wells		Ig in Supernatants				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	6.2×10^{-7}	5.0×10^{-6}	8	8	3	1	nd	nd	0
B	2.0×10^{-7}	8.0×10^{-7}	3	3	1	1	nd	nd	0
C	$<4.8\times10^{-8}$	$<1.5\times10^{-7}$	0	0					
D	6.0×10^{-6}	3.0×10^{-5}	106	97	92	5	nd	nd	0
E	6.7×10^{-6}	1.6×10^{-5}	125	120	88	27	nd	nd	0
F	$>3.0\times10^{-5}$	$>8.0\times10^{-5}$	420	316	216	100	nd	nd	0
G	1.0×10^{-6}	2.8×10^{-6}	8	8	8	0	nd	nd	0

	Fusion efficiency: Day 2		wells		Ig in Supernatant				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	1.2×10^{-6}	3.6×10^{-6}	12	12	5	4	nd	nd	0
B	5.1×10^{-6}	1.4×10^{-5}	56	56	34	18	nd	nd	0
C	3.3×10^{-7}	7.5×10^{-7}	0	0					
D	2.5×10^{-7}	6.0×10^{-7}	3	3	2	0	nd	nd	0
E	8.3×10^{-8}	2.0×10^{-7}	0	0					
F	5.0×10^{-7}	1.3×10^{-6}	4	4	3	1	nd	nd	0
G	$<2.3\times10^{-8}$	$<2.0\times10^{-7}$	0	0					

	Fusion efficiency: Day 3		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	$<1.3\times10^{-7}$	$<2.0\times10^{-7}$	0	0					
B	3.3×10^{-7}	6.7×10^{-7}							
C	$<1.7\times10^{-7}$	$<3.0\times10^{-7}$							
D	$<1.4\times10^{-7}$	$<3.3\times10^{-7}$							
E	$<1.1\times10^{-7}$	$<2.9\times10^{-7}$							
F	1.0×10^{-6}	3.0×10^{-6}							
G	$<1.7\times10^{-7}$	$<5.0\times10^{-7}$							

	Fusion efficiency: Day 4		wells		Ig in Supernatants:				
	total cells:	blasts:	scored:	assayed:	κ	λ	IgM	IgG	DT/KLH
A	$<1.7\times10^{-7}$								
B	$<2.0\times10^{-7}$								
C	$<1.7\times10^{-7}$								
D	$<1.3\times10^{-7}$								
E	$<1.3\times10^{-7}$								
F	$<1.7\times10^{-7}$								
G	$<1.7\times10^{-7}$								

5. DISCUSSION AND CONCLUSIONS

During the past 12 months almost 3,000 hybridoma supernatants containing >5 μg/ml of immunoglobulin have been generated and screened for DT–specific binding activity. Since none has shown the sought–after activity, yet the normal human population is chronically immune to this antigen, we have been forced to conclude that there is likely a defect in the human tumor cell line WI–L2–729–HF_2 which prohibits the functional expression of the normal B–cell immunoglobulin.

Although the WI–L2–729–HF_2 tumor cell line is clearly the best available in terms of high frequency production of hybridomas that stably produce immunoglobulin, we have reasons to suspect that the immunoglobulin (Ig) genes expressed in the cell line (and perhaps others), are quite different to the configuration found in murine myelomas; indeed it is recognized that all of the tumor B–cell parental lines used in producing human hybridomas are early stage B–cells, and that the only other technique for immortalizing human B–cells (namely Epstein–Barr Virus, EBV) also has a predilection for transforming early stage B–cells. Moreover, there is considerable experience to suggest that human myelomas are difficult to maintain in the immunoglobulin–secreting stage in culture; and in the murine system what little is known about early stage B–cell tumor lines suggests that they have quite different regulatory mechanisms operating as compared with the mature myelomas. experience has now forced us to concede that the above argument likely correct. We must, therefore, put aside the rare successes of the past and concentrate on the majority of "failures" if we are to proceed rationally toward a usable human hybridoma system.

In our laboratory Dr. J. Heitzmann has begun to accumulate evidence that further confirms our suspicions and, more importantly, offers a line of experimentation that should eliminate many of the major drawbacks to the WI–L2–729–HF_2 cell line. Since Dr. J. Heitzmann's work is not yet published, it is inappropriate to describe that work in any detail here. We are, nonetheless, confident that the human hybridoma system is workable, and the original arguments which made human hybridomas a sufficiently valuable technique to warrant a major investment remain as cogent as ever.

6. LITERATURE CITED

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